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Scope: The discovery of p73, a gene that in certain experimental conditions behaves just like p53, requires us to determine what role it plays in breast cancer, whether a crosstalk exists between p73 and p53 actions and to clearly delineate the differences and similarities between these two genes concerning their biological role and signaling pathways. Our understanding of p53's role in breast cancer has been made hazier again by the advent of p73's discovery. For example, it has already been shown that p73's transactivation and apoptotic function is inhibited by tumor-derived p53 mutants. This opens the possibility that the phenotype of mutant p53 tumor cells might in fact be due to an interference with normal p73 function. Conversely, certain p73 isoforms could be dominant negative over p53 in heterotypic interactions. In this context it was already shown among the various p73 isoforms that, depending on the combination, synergistic or antagonistic biologic effects can ensue. This scenario could be the underlying explanation why only 30 % of breast cancers have mutated their p53 gene. In this case, dominant negative p73 isoforms, when deregulated in breast cancer, could interfere with p53 and p73-mediated growth suppression.

About 40% of breast cancers overexpress p73, indicating its rolein breast cancer tumorigenesis. A better insight into p73's function will add greatly to our understanding of its role in this disease.

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In vivo role of the p73 gene in breast cancer

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Ute M. Moll, M.D.
Second Year Progress Report

Scope: The discovery of p73, a gene that in certain experimental conditions behaves just like p53, requires us to determine what role it plays in breast cancer, whether a crosstalk exists between p73 and p53 actions and to clearly delineate the differences and similarities between these two genes concerning their biological role and signaling pathways. Our understanding of p53's role in breast cancer has been made hazier again by the advent of p73's discovery. For example, it has already been shown that p73's transactivation and apoptotic function is inhibited by tumor-derived p53 mutants. This opens the possibility that the phenotype of mutant p53 tumor cells might in fact be due to an interference with normal p73 function. Conversely, certain p73 isoforms could be dominant negative over p53 in heterotypic interactions. In this context it was already shown among the various p73 isoforms that, depending on the combination, synergistic or antagonistic biologic effects can ensue. This scenario could be the underlying explanation why only 30 % of breast cancers have mutated their p53 gene. In this case, dominant negative p73 isoforms, when deregulated in breast cancer, could interfere with p53 and p73-mediated growth suppression.

About 40% of breast cancers overexpress p73, indicating its rolein breast cancer tumorigenesis. A better insight into p73's function will add greatly to our understanding of its role in this disease.

Background:

Role of p53 in human cancer. The p53 tumor suppressor is absolutely crucial in cancer. Loss of p53 function is a preeminent finding in all human cancers, whether directly through mutation of itself (1) or its upstream activator p14ARF (2), indirectly through inhibition by its antagonist mdm2 (3) or p53's inability of nuclear retention (4-6). In normal cells, p53 controls a stress response pathway following a broad range of DNA damage and inappropriate oncogenic signals. Reintroduction of p53 protein, a homotetrameric transcription factor, into p53-defective tumor cells causes apoptosis or cell cycle arrest and enhances the cell's susceptibility to killing by anticancer agents.

The p73 gene. Despite p53's central role in carcinogenesis, no related genes were known for 20 years. In 1997, two novel family members were identified and termed p73 and p63 (7, 8). P73 shares 63% identity with the DNA-binding region of p53 including conservation of all DNA contact residues, 38% with the tetramerization domain and 29% with the transactivation domain. The p73 gene is ancestral to p53 (7, 8). In contrast to p53, human p73 produces at least six C-terminal splice variants (α, β, γ, δ, ε, ϕ) (7, 9, 10).

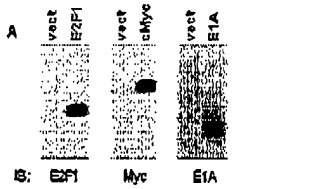
P73 function in overexpression system. When ectopically overexpressed in cell culture, p73 α and β closely mimic p53 activities. P73 β, and to a lesser extent p73 α, transactivate many p53-responsive promoters, although relative efficiency differences on a given promoter seem to be common (11-13). Like p53, p73 forms a complex with p300/CBP which mediates transcription by p73 (14). Ectopic p73 also promotes apoptosis irrespective of the p53 status (7). Moreover, overexpression of p73 α, β and δ suppresses focus formation, while p73 γ does not (9, 11). The suppressor activity of isoforms ε and ϕ have not been determined.

P73 is not a classic Knudson-type tumor suppressor. P73 maps to chrom 1p36.3 which frequently undergoes loss of heterozygosity (LOH) in breast cancer, neuroblastoma and several other cancers (7). This fact, in conjunction with the functional similarity to p53, originally led D. Caput, the discoverer of p73, to propose that p73 is a tumor suppressor gene (7). However, based on newer data the mode of p73's role in tumorigenesis is unclear. Current genetic data exclude p73 to be a Knudson-type tumor suppressor, targeted to undergo loss of expression during tumorigenesis. To date, mutations in the p73 gene are vanishingly rare. Moreover, imprinting of the p73 locus, initially thought to be an epigenetic explanation to satisfy the 2 hit hypothesis (since it would only require one hit of LOH against the transcribed allele), is less common and if present, varies from tissue to tissue (10, 15, 16, 17). In fact, in lung, esophageal and renal carcinoma, the second p73 allele is specifically activated in the tumor compared to the normal tissue of origin (loss of imprinting) (18-20). Also, p73 protein is not targeted by the viral

In the last 12 months we focused our efforts on fulfilling Aim IB:
Determine if cellular oncogenes induce p73 protein(s) and if this is signaled through p19ARF.

Progress:

Oncogenes and p14ARF Induce and Activate Endogenous p73 Protein. Defining the upstream pathways that signal to p73 will be crucial for understanding its biological role. We asked whether oncogenes can induce and activate endogenous p73. We show that p73 α and β proteins are upregulated in p53-deficient H1299 cells in response to overexpressed E2F1, cMyc and E1A (Fig.1). The oncogene-mediated p73 accumulation is stronger and broader than the reported p73 response after cisplatin. E2F1, cMyc and E1A-mediated p73 upregulation leads to activation of p73 transcription function, as shown by the endogenous p73 targets p21 and HDM2 (Fig.2), and by p73-responsive reporter activity which is inhibited by a dominant negative mutant of p73 (p73DN). Importantly, oncogene-mediated activation of endogenous p73 induces apoptosis in Saos2 cells, which is largely abrogated by p73DN, indicating p73 dependence (Fig.3). In contrast, among seven stable H1299 clones overexpressing cMyc, p73 accumulates but largely fails to be active, indicating that in p53-deficient tumor cells with activated oncogenic pathways, clonal outgrowth favors loss of p73 function. Taken together, this data shows that oncogenes can signal to p73 *in vivo*. Moreover, our data provide a mechanism for the fact that a broad spectrum of human tumors, with their frequent deregulation of oncogenes such as E2F1 and myc, overexpress p73. See Reference 39.



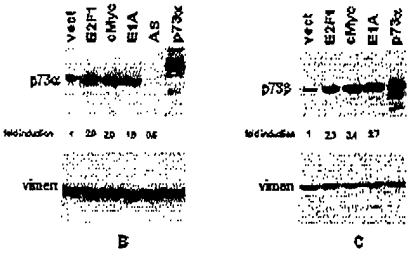


Fig. 2

Fig 1. *P73 α and β proteins are induced in response to cellular and viral oncogenes.* **A.** Immunoblots of H1299 cells after transient transfection with empty vector or expression vectors for E2F1, cMyc and E1A. Blots were developed with the indicated antibodies. **B.** Immunoblots of H1299 cells probed with polyclonal p73 α antibody, or C. with p73 β antibody (GC15) after transient transfection with empty vector (vect) or expression vectors for E2F1, cMyc and E1A. Fold induction of p73 protein levels are indicated. HA-tagged p73 α and p73 β transfected into H1299 cells serve as positive controls, respectively. SK-N-AS cells are used as negative control. Membranes were reblotted for vimentin to assure equal loading.

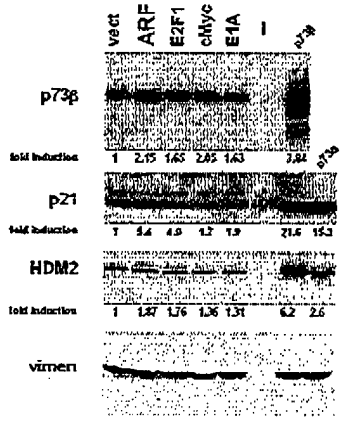


Fig 2. *Oncogene and p14 ARF -mediated p73 induction leads to functional activation of p73.* H1299 cells were transiently transfected with empty vector (vect) or the same amount of the indicated expression plasmids (2 μg). Total cell extracts were immunoblotted for p73 β, p21 and HDM2. Fold induction of p73 target protein levels are indicated. Membranes were reblotted for vimentin to assure equal loading (about 30 μg per lane). H1299 cells directly transfected with 2 μg of HA-p73 α and β expression plasmids, respectively, are shown for comparison (last two lanes). Four independent experiments gave similar results.

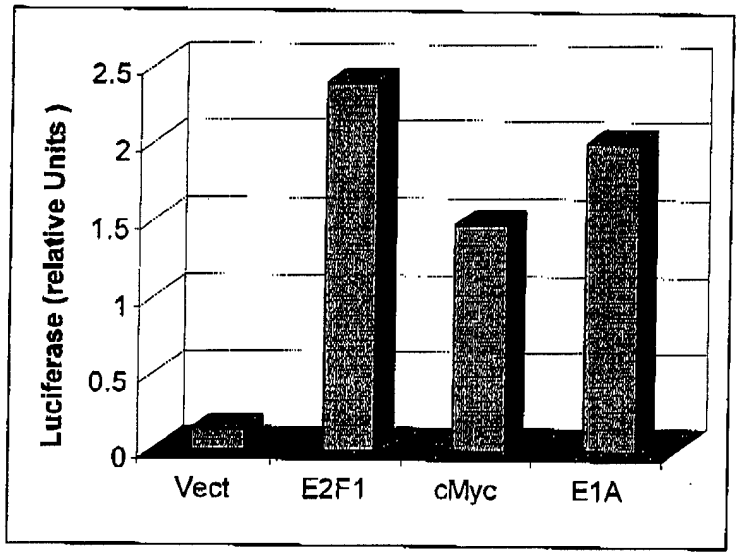


Fig. 3 Oncogene-mediated induction of endogenous p73 leads to functional activation of p73.

Oncogenc-mediated activation of the p73 responsive reporter PG13-Luc. H1299 cells were transiently transfected with empty vector or the indicated expression plasmids (400 ng each) together with 80 ng of PG13-Luc and 8 ng of renila luciferase. Luciferase activity was measured 24 h later and standardized for renila activity. E2F1 exhibited a 16.5-fold, cMyc a 10.3-fold and E1A a 13.9-fold induction compared to vector controls. Results are the average ± s.d. of 3 independent experiments.

Oncogene-mediated activation of p73 induces apoptosis in p53-deficient tumor cells.
Importantly, oncogene-mediated activation of endogenous p73 induces apoptosis in Saos2 cells, which is largely abrogated by p73DN, indicating p73 dependence (Fig.4). In contrast, among seven stable H1299 clones overexpressing cMyc, p73 accumulates but largely fails to be active for p73 reporter activity and p21and HDM2 induction, suggesting that in p53-deficient tumor cells with activated oncogenic pathways, clonal outgrowth favors loss of p73 function (data not shown). Taken together, this data shows that oncogenes can signal to p73 *in vivo*. Moreover, our data provide a framework for the fact that a broad spectrum of human tumors, with their frequent deregulation of oncogenes such as E2F1 and myc, overexpress p73. See Reference 39.

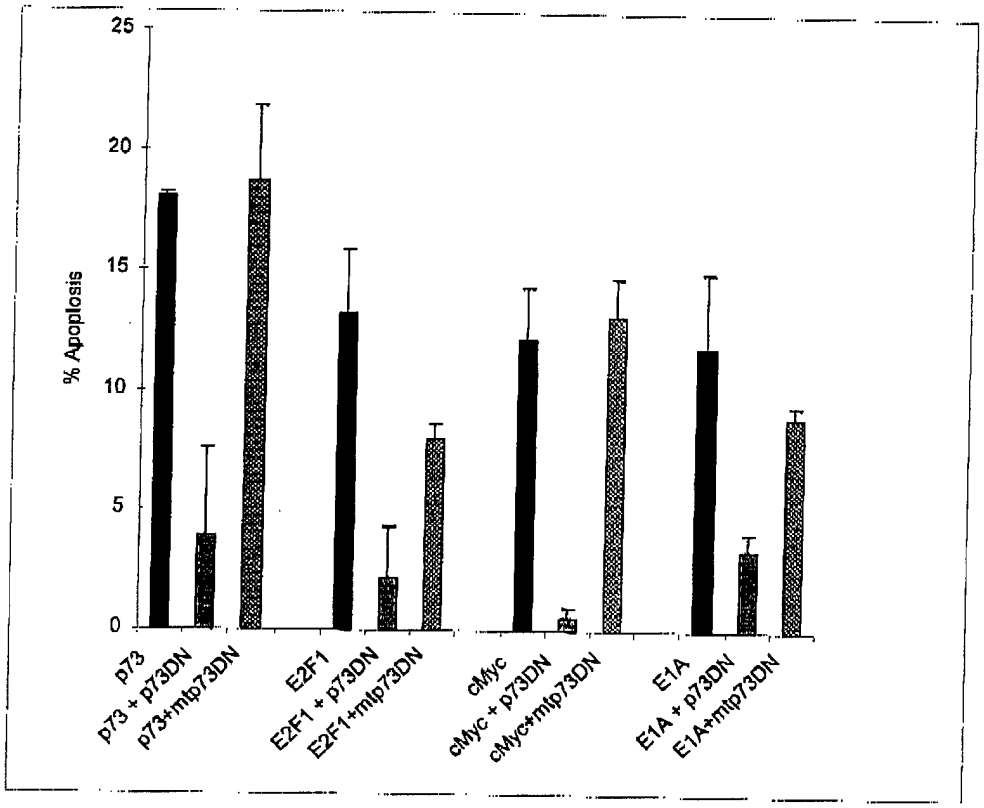


Fig 4. *Oncogene-mediated activation of p73 induces apoptosis in p53-deficient tumor cells.* Saos-2 cells in duplicate wells were transfected with expression plasmid (150 ng) plus empty vector (350 ng) (black columns), or expression plasmid (150 ng) plus p73DN mutant (350 ng) (grey columns) or), or expression plasmid (150 ng) plus a functionally inactive p73DN mutant L371P (350 ng) (striped columns). After 24 h cells were processed in parallel for TUNEL and for immunofluorescence to determine expression. The percentage of apoptosis of transfected cells is shown after correction for background with vector alone (500 ng per well). The results represent the average ± s.d. of 3 independent experiments.

Summary: We have shown that in transient transfection systems, the three oncogenes E2F1, cMyc and E1A can indeed upregulate and transactivate endogenous p73. Moreover, p73 can mediate oncogene-induced apoptosis in p53-deficient tumor cells. This suggest that in certain circumstances, p73 can at least partially compensate for p53 in human tumors. Whether or not this signaling is regulated through p14ARF will need further investigation

This work is ready for submission to a scientific journal.

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